

CERTIFICATION REPORT

**The certification of different mass fractions of
MON-04032-6 in soya bean powder**

**Certified Reference Materials
ERM[®]-BF410ap, ERM[®]-BF410bp, ERM[®]-BF410cp,
ERM[®]-BF410dp and ERM[®]-BF410ep**



European Commission
Joint Research Centre
Directorate F – Health, Consumers and Reference Materials

Contact information
Reference materials sales
Address: Retieseweg 111, 2440 Geel, Belgium
E-mail: jrc-rm-distribution@ec.europa.eu
Tel.: +32 (0)14 571 705

JRC Science Hub
<https://ec.europa.eu/jrc>

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JRC 107119

EUR 28665 EN

ISBN 978-92-79-70107-8 (PDF)
ISSN 1831-9424 (online)
doi: 10.2760/341185

Luxembourg: Publications Office of the European Union, 2017
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Abstract

This report describes the production of a set of Certified Reference Materials (CRMs), ERM-BF410ap, bp, cp, dp and ep, which are certified for their GTS 40-3-2 soya bean (unique identifier MON-04032-6) event mass fractions. These materials were produced following ISO Guide 34:2009 and are certified in accordance with ISO Guide 35:2006. The materials are intended for the calibration or quality control of real-time PCR measurements to identify GTS 40-3-2 soya bean and quantify its mass fraction. As with any reference material, they can also be used for establishing control charts or for carrying out validation studies. The CRMs were accepted as European Reference Material (ERM®) after peer evaluation by the partners of the European Reference Materials co-operation.



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ERM[®]-BF410dp and ERM[®]-BF410ep**

B. Dimitrievska, A.M. Kortekaas, J. Seghers, H. Leys,
Ph. Corbisier, S. Trapmann

European Commission, Joint Research Centre
Directorate F – Health, Consumers and Reference Materials
Geel, Belgium

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Summary

This report describes the production of a set of Certified Reference Materials (CRMs), ERM-BF410ap, bp, cp, dp and ep, which are certified for their GTS 40-3-2 soya bean (unique identifier MON-Ø4Ø32-6) event mass fractions. These materials were produced following ISO Guide 34:2009 [1] and are certified in accordance with ISO Guide 35:2006 [2].

Genetically modified (GM) soya bean seeds of the GTS 40-3-2 event and seeds from a non-GM soya bean variety were milled to obtain GM and non-GM seed powders with a similar particle size distribution. Mixtures of non-GM and GM soya bean seed powder were prepared gravimetrically.

The certified values were obtained from the gravimetric preparations, taking into account the water mass fractions of the two powder materials and the genetic purity with respect to the GTS 40-3-2 soya bean. The certified values were confirmed by event-specific real-time PCR as an independent verification method (measurements were within the scope of accreditation to ISO/IEC 17025:2005 [3]).

The uncertainties of the certified values were estimated in accordance with the Guide to the Expression of Uncertainty in Measurement (GUM) [4] and include uncertainties relating to possible inhomogeneity (Section 4), instability (Section 5) and characterisation (Section 6).

The materials are intended for the calibration or quality control of real-time PCR measurements to identify GTS 40-3-2 soya bean and quantify its mass fraction. As with any reference material, they can also be used for establishing control charts or for carrying out validation studies. The CRMs are available in glass bottles containing at least 1 g of dried soya bean seed powder, sealed under an atmosphere of argon. The minimum amount of sample to be used for extraction of the DNA is 200 mg.

The CRMs were accepted as European Reference Material (ERM®) after peer evaluation by the partners of the European Reference Materials co-operation.

The following values were assigned:

	GTS 40-3-2 soya bean mass fraction ¹⁾	
	Certified value [g/kg]	Uncertainty [g/kg] ⁵⁾
ERM-BF410ap	< 0.09 ²⁾	-
ERM-BF410bp	> 985 ³⁾	-
ERM-BF410cp	1.00 ⁴⁾	0.10
ERM-BF410dp	10.0 ⁴⁾	0.6
ERM-BF410ep	100 ⁴⁾	5

1) Genetically modified soya bean with the unique identifier MON-Ø4Ø32-6-soya bean.

2) The certified reference material has been produced from conventional, non-modified soya bean seeds. No contamination was detected in this material when using an event-specific real-time polymerase chain reaction assay targeting the GTS 40-3-2 soya bean event. The limit of detection (LOD) was 0.09 g/kg. With 95 % confidence, the true GTS 40-3-2 soya bean mass fraction of the material is below 0.09 g/kg. The certified value is traceable to the International System of Units (SI).

3) This certified reference material was produced from genetically modified GTS 40-3-2 soya bean seeds. The certified value is based on the genetic purity of the soya bean powder with regard to GTS 40-3-2 soya bean. In total 201 seeds were tested individually for the presence of the GTS 40-3-2 soya bean event. All seeds tested positive. With 95 % confidence, the true GTS 40-3-2 soya bean mass fraction of the material is above 985 g/kg. The certified value is traceable to the International System of Units (SI).

4) This certified value is based on the masses of dried genetically modified GTS 40-3-2 soya bean powder and dried non-modified soya bean powder that were mixed, taking into account their respective genetic purity with regard to GTS 40-3-2 soya bean and their respective water content. The certified value is traceable to the International System of Units (SI).

5) The uncertainty is the expanded uncertainty with a coverage factor $k = 2$ corresponding to a level of confidence of about 95 %, estimated in accordance with ISO/IEC Guide 98-3, Guide to the Expression of Uncertainty in Measurement (GUM:1995), ISO, 2008.

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Glossary

ANOVA	Analysis of variance
Cq	Quantification cycle (also referred to as threshold cycle, Ct)
CRM	Certified reference material
CTAB	Cetyltrimethylammonium bromide
DNA	Deoxyribonucleic acid
EC	European Commission
ERM®	Trademark of European Reference Materials
EU	European Union
EURL-GMFF	European Union Reference Laboratory for Genetically Modified Food and Feed
<i>g</i>	Relative centrifugal force
GM	Genetically modified
GMO	Genetically modified organism
GUM	Guide to the Expression of Uncertainty in Measurements
EDTA	Ethylenediaminetetraacetic acid
EPSPS	5-enolpyruvylshikimate-3-phosphate synthase
IEC	International Electrotechnical Commission
ISO	International Organization for Standardization
JRC	Joint Research Centre
<i>k</i>	Coverage factor
LOD	Limit of detection
MS_{between}	Mean of squares between-unit from an ANOVA
MS_{within}	Mean of squares within-unit from an ANOVA
<i>n</i>	Number of replicates per unit
<i>N</i>	Number of samples (units) analysed
n.a.	Not applicable
n.c.	Not calculated
PCR	Polymerase chain reaction
PSA	Particle size analysis
rel	Index denoting relative figures (uncertainties etc.)
RM	Reference Material
rpm	Revolutions per minute
RT	Room temperature
<i>s</i>	Sample standard deviation
$s_{\bar{x}}$	Standard deviation of the estimate of the mean (also referred to as standard error of the estimate of the mean)
s_{bb}	Between-unit standard deviation; an additional index "rel" is added as appropriate
SI	The International System of Units
s_{rel}	Relative standard deviation (also referred to as RSD)
s_{wb}	Within-unit standard deviation; an additional index "rel" is added as appropriate
<i>t</i>	Time
t_i	Time point for each replicate
TaqMan®	<i>Thermus aquaticus</i> (Taq) DNA polymerase-based technology for fluorescent signal generation in real-time PCR
TE	Tris-EDTA
<i>u</i>	Standard uncertainty
<i>U</i>	Expanded uncertainty
u_{bb}^*	Standard uncertainty related to a maximum between-unit inhomogeneity that could be hidden by the intermediate precision of the method; an additional index "rel" is added as appropriate

u_{bb}	Standard uncertainty related to a possible between-unit inhomogeneity; an additional index "rel" is added as appropriate
u_{char}	Standard uncertainty of the material characterisation; an additional index "rel" is added as appropriate
u_{CRM}	Combined standard uncertainty of the certified value; an additional index "rel" is added as appropriate
U_{CRM}	Expanded uncertainty of the certified value; an additional index "rel" is added as appropriate
u_{lts}	Standard uncertainty of the long-term stability; an additional index "rel" is added as appropriate
u_{sts}	Standard uncertainty of the short-term stability; an additional index "rel" is added as appropriate
V-KFT	Volumetric Karl Fischer Titration
\bar{x}	Arithmetic mean
\bar{y}	Mean of all results of the homogeneity study
ν	Degrees of freedom
w/v %	Weight / volume percentage
v/v %	Volume / volume percentage

1 Introduction

1.1 Background: need for the CRM

The European Union has legislation which regulates the placing on the market of any food or feed which consists of, contains, or is produced from genetically modified organisms (GMOs). These items are referred to as genetically modified food and feed and require authorisation for marketing in the European Union. They are also required to be labelled if they contain more than 0.9 % of GMOs [5]. This labelling threshold is applicable for the adventitious presence of GMOs, whilst GMOs that are intentionally added need to be labelled independently from any threshold. However, feed may contain 0.1 (m/m) % of a GMO for which an authorisation process is pending, or for which authorisation in the EU has expired [6]. These thresholds require the development and validation of reliable methods for GMO quantification, and the production of reference materials for calibration or quality control of these methods.

Monsanto (St. Louis, Missouri, US) developed the genetically modified (GM) GTS 40-3-2 soya bean event (unique identifier code MON-Ø4Ø32-6, following Commission Regulation (EC) No 65/2004 [7]) as a transgenic herbicide tolerant crop. The GTS 40-3-2 soya bean event was developed by *Agrobacterium tumefaciens* mediated transformation. The event contains the gene coding the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) that gives the plant resistance to glyphosate, the active ingredient in the herbicide Roundup® [8]. The new production series, which is the subject of this certification report, received the code ERM-BF410p with 'p' indicating the replacement batch. It is composed of five CRMs containing different mass fractions of GTS 40-3-2 soya bean. Unlike the previous productions, in this batch the codes used for the different concentrations of the mass fraction of GTS 40-3-2 soya bean followed the new labelling pattern where the ERM-BF410ap and ERM-BF410bp are the pure non-GM and GM materials, and ERM-BF410cp, dp and ep are 0.1 %, 1 % and 10 % materials, respectively.

1.2 Choice of the material

The set of CRMs ERM-BF410p was produced from milled GM and non-GM seeds. Seeds (in contrast to the grains) were selected as the source of raw material because of their high degree of purity.

1.3 Design of the CRM project

The genetic purity with respect to the GTS 40-3-2 soya bean event of the non-GM and GM soya bean seeds has been investigated.

Alongside the pure non-GM material ERM-BF410ap and the pure GM material ERM-BF410bp, mixtures of non-GM and GM soya bean powder were prepared gravimetrically. The first mixed material ERM-BF410ep was prepared by mixing pure GM with non-GM soya bean powder. ERM-BF410dp was prepared by further dilution of ERM-BF410ep, and ERM-BF410cp was prepared by further dilution of ERM-BF410dp, in both cases with non-GM soya bean powder.

The different mass fractions of ERM-BF410p were certified using a gravimetric approach, the details of which are described in Section 6.

2 Participants

2.1 Provider of raw material and quantification method

Monsanto (St. Louis, Missouri, US) provided the raw materials.

Monsanto initially provided the event-specific real-time PCR method under a confidential agreement with JRC. Since 2007, it is validated and published by the EURL-GMFF [9].

2.2 Project management, processing, analytical measurements and evaluation

European Commission, Joint Research Centre, Directorate F – Health, Consumers and Reference Materials

(accredited to ISO Guide 34 for production of certified reference materials, BELAC No. 268-RM, and to ISO/IEC 17025 for GMO quantification, BELAC No. 268-TEST)

3 Material processing and process control

3.1 Origin of the starting material

Monsanto (St. Louis, Missouri, US), supplied the JRC in Geel, Belgium (JRC-Geel) with non-GM soya bean seeds and GTS 40-3-2 soya bean seeds to prepare candidate CRMs. According to the information provided by Monsanto, the GTS 40-3-2 soya bean seeds (trade name - Roundup Ready soya bean) are homozygous. After the arrival, the seeds were stored at $(4 \pm 3) ^\circ\text{C}$ in the dark until processing.

The genetic purity with respect to the GTS 40-3-2 soya bean event of the GM soya bean seeds was assessed at JRC-Geel by analysing 201 randomly selected seeds for the presence of the GTS 40-3-2 soya bean GM event. Genomic DNA was extracted from 171 plants grown from individual seeds using the DNeasy Plant Mini kit (Qiagen, Venlo, NL). The event-specific real-time PCR method to detect the GTS 40-3-2 soya bean event is internationally validated by the European Union Reference Laboratory for Genetically Modified Food and Feed (EURL-GMFF), and it is published on the EURL homepage [9]. Genomic DNA extracted from pure GTS 40-3-2 soya bean powder was used as positive control using the CTAB method (Annex A). Amplification and detection was performed on a QuantStudio 7 real-time PCR system following the TaqMan® Universal PCR Master Mix protocol (Thermo Fisher Scientific, Foster City, CA, USA) [10]. Thirty vital seeds were tested for the presence of GTS 40-3-2 soya bean event using the lateral flow strip tests (AgraStrip® RUR Strip Test Seed & Leaf - Trait Test Kit) that detects the CP4 EPSPS protein produced by a gene, derived from *Agrobacterium sp.* strain CP4. The results showed that all the GM seeds tested gave a signal for the presence of the GTS 40-3-2 soya bean event. A possible presence of the stacked event 305423 x GTS 40-3-2 soya bean detected with these lateral flow strips was excluded because of the negative result of the 305423 event shown on the pre-spotted PCR 96-well plate used for simultaneous detection of 13 GM specific events. Statistical analysis of the 201 measurements (Poisson distribution for rare events) revealed that the GM soya bean seed batch had a genetic purity $> 98.5 \%$ (95 % level of confidence). This was taken into consideration during the estimation of the uncertainties associated with the certified values of the CRMs (Section 6.2).

The genetic purity of the non-GM seed batch with respect to the GTS 40-3-2 soya bean event was investigated using the processed seed powder. Five bottles of ERM-BF410ap were randomly selected and the DNA was extracted from two samples taken from each bottle (extraction replicates, $N = 5$, $n = 2$). Each DNA extract was then analysed in 3

replicates by real-time PCR method, with a limit of detection (LOD) of 0.09 g/kg. This analysis did not detect the GTS 40-3-2 soya bean event (Section 3.4). The LOD of the event-specific real-time PCR method was taken into consideration when the certified value of ERM-BF410ap was calculated (Section 7).

3.2 Processing and process control

All soya bean seeds received by the JRC-Geel were rinsed with water, drained, and dried on trays in the drying chamber of a freeze-dryer at 25 °C for 20 h (Epsilon 2-100D, Martin Christ, Osterode, DE).

Approximately 20 kg of non-GM soya bean seeds and 10 kg of GTS 40-3-2 soya bean event seeds were used for the production of the ERM-BF410p.

The GM and non-GM base materials were processed separately into powders. Cross-contamination between them and contamination with foreign DNA were avoided by treating all the contact surfaces with DNA degrading solution (DNA-Erase™, MP Biomedicals, Irvine, CA, USA) before exposure to the materials and using clean laboratory clothing. An in-house validation study had previously shown that the solution degraded DNA effectively under the given conditions.

The soya bean seeds were frozen overnight in liquid nitrogen in approximately 4 kg portions in stainless steel containers and were subsequently milled using a cryo-grinding vibrating mill (Palla mill, KHD, Humboldt-Wedag, Köln, DE). The mill was maintained below -90 °C throughout the process. The feeding speed of the mill was optimised to ensure that the seeds were milled to the required particle size. After milling, the powder was maintained at (4 ± 3) °C. The GM and non-GM powders were then separately sieved with a 710 µm stainless steel mesh on a sieving machine equipped with an ultra-sound sieving aid (Russel Finex, London, UK). The remaining powder from each base material was mixed in a DynaMIX CM200 (WAB, Basel, CH) for 1 h to homogenize the distribution of the different types of soya bean seed tissues, since it is known that the milling and sieving processes result in separation of the various seed tissues from each other.

The residual water mass fractions of the non-GM and GM powders were measured by volumetric Karl Fischer titration (V-KFT, 841 KFD Titrand, Metrohm, Herisau, CH), as (65.1 ± 4.2) g/kg and (67.9 ± 4.4) g/kg respectively ($N = 1$, $n = 3$), with the expanded uncertainty calculated using a coverage factor $k = 2$. To facilitate gravimetric mixing, the water content of the powders was further reduced by drying overnight under vacuum, in the freeze-dryer at 25 °C. The final water mass fractions of the non-GM powder and the GM powder were measured as (25.6 ± 1.7) g/kg and (15.5 ± 1) g/kg, respectively ($N = 1$, $n = 3$), with the expanded uncertainty calculated using a coverage factor $k = 2$, (Table 1).

Also, the particle size distribution for both powders was measured based on their deconvoluted laser diffraction patterns (PSA, Sympatec, Clausthal-Zellerfeld, DE) and were then compared (Figure 1). The cumulative volume distribution of the particles derived from laser diffraction data is based on their equivalent spherical diameter, i.e. the diameter of the particles derived from the volume occupied upon their rotation. The mean particle diameter of the non-GM and GM powder materials was $118.2 \mu\text{m} \pm 17.1 \mu\text{m}$ (s) and $121.3 \mu\text{m} \pm 3.0 \mu\text{m}$ (s), respectively. However, since most particles are not perfectly spherical, the calculated volume of the particles based on their diameter will overestimate the mean particle size. Therefore, a three-point specification of the particle size distribution ($N = 1$, $n = 3$) was calculated, consisting of the equivalent sphere diameters where 10 %, 50 % and 90 % of the total volume distribution have a smaller particle size (Table 1). These size classes are denoted as X_{10} , X_{50} and X_{90} , respectively. A *t*-test showed with 95 % confidence that there was no significant difference between the X_{10} , X_{50} , X_{90} values and between the mean particle diameter of the non-GM and GM soya bean powders. It was concluded that the non-GM and GM powder materials were sufficiently similar with respect to their particle size distribution

and they could be processed further without introducing a bias which could subsequently affect the extractability of the DNA.

Table 1: The water mass fraction determined by V-KFT and additionally the particle diameter and particle size distribution based on the deconvoluted laser diffraction patterns of the powder materials

Powder material	Water mass fraction [g/kg]		Mean particle diameter [μm]		Particle size distribution X10 [μm]		Particle size distribution X50 [μm]		Particle size distribution X90 [μm]	
	\bar{x}	U	\bar{x}	s	\bar{x}	U	\bar{x}	U	\bar{x}	U
Non-GM powder	25.6 ¹⁾	1.7	118.2 ²⁾	17.1	17.6 ³⁾	3.8	101.6 ³⁾	18.0	242.7 ³⁾	50.5
GM powder	15.5 ¹⁾	1.0	121.3 ²⁾	3.0	17.8 ³⁾	3.8	104.6 ³⁾	18.6	248.4 ³⁾	51.7

¹⁾ Mean of one sample ($N = 1$, $n = 3$). The associated expanded uncertainty (U) with a coverage factor $k = 2$ has been estimated during validation of the V-KFT method on soya bean powder.

²⁾ Mean of one sample ($N = 1$, $n = 3$) with the sample standard deviation

³⁾ Mean of one sample ($N = 1$, $n = 3$). Given are the equivalent sphere diameters for which 10 %, 50 % or 90 % of the particles in the volume distribution have a smaller particle size. The associated expanded uncertainty (U) with a coverage factor of $k = 2$ has been estimated during validation of the particle size measurement method.

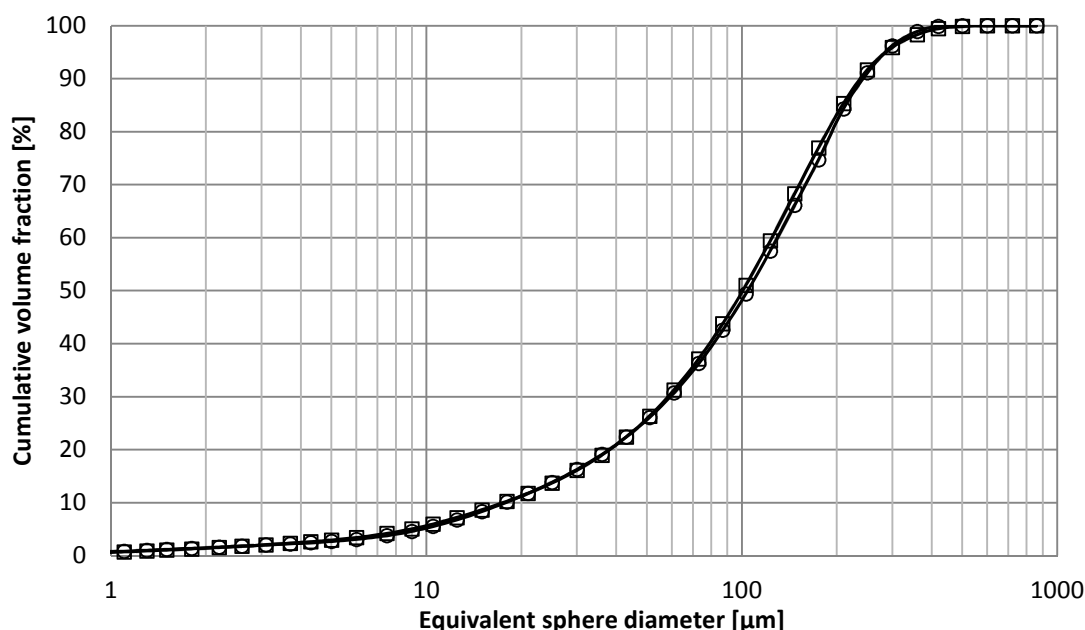


Figure 1: Volume-based cumulative distribution of equivalent sphere diameters in the GM powder (o) and non-GM powder (□) analysed by laser diffraction ($N = 1$, $n = 3$). The total particle volume for each material is set as 100 %.

The milled base materials were used to prepare the blank material for GTS 40-3-2 soya bean (non-GM soya bean seed powder), the pure GM GTS 40-3-2 soya bean material and three mixtures at nominal mass fraction levels of 1, 10 and 100 g/kg GTS 40-3-2 soya bean event. The term "nominal" is used for the target value during the processing whereas the value assigned after completion of the certification process is called certified value.

All the materials were treated according to the same procedure and strict measures were taken to avoid cross-contamination. The powder materials were weighed using a calibrated balance (MSU-8202-S, Sartorius) with an intermediate precision, determined during calibration and expressed as standard uncertainty (u), of 0.02 g. Calibration of the balance is

performed on an annual basis by an external company (accredited under ISO/IEC 17025). The performance of the balance was verified before use on a daily basis by using in-house reference weights. The masses of the non-GM and GM powders, which are theoretically needed to reach a certain nominal mass fraction, were calculated corrected for their respective water content. Portions of the powder materials were weighed into a container and mixed for 1 h by using a Dyna-MIX CM 200 (WAB, Basel, CH). The material with a nominal GTS 40-3-2 soya bean mass fraction of 100 g/kg was produced by mixing pure GM with pure non-GM powder materials. Similarly, the material with a nominal GTS 40-3-2 soya bean mass fraction of 10 g/kg was produced by further dilution of the 100 g/kg GM powder with pure non-GM powder and the material with a nominal mass fraction of 1 g/kg was produced by further dilution of the 10 g/kg GM powder with pure non-GM powder. At each mixing step, the water mass fraction of the materials was taken into account (Table 6). During the certification process, the gravimetric preparation was the basis for the calculation of the certified GTS 40-3-2 soya bean mass fraction for the three powder mixtures (Section 6).

An automatic filling device (All-Fill Sandy, UK) was used to fill the powders into 10 mL amber glass bottles. To avoid cross contamination the equipment was cleaned between the two mass fraction levels and the first 30 bottles of each batch were discarded as an additional precaution. The blank material was filled first, followed by the mixtures with increasing mass fraction with the pure GM material filled last. Lyophilisation inserts were automatically placed in the bottle necks. The bottles were then placed in a freeze-dryer (Epsilon 2-100D, Martin Christ, Osterode, DE) to provide an argon atmosphere, and were closed inside the freeze-dryer with the help of a hydraulic device. Capping and labelling took place in a capping and labelling assembly from Bausch & Ströbel and BBK, respectively (Ilshofen and Beerfelden, both in Germany). Colour-coded caps were used to facilitate the identification of the different mass fraction levels of GTS 40-3-2 soya bean event: nominal 0 g/kg = silver (BF410ap), nominal 1000 g/kg = black (BF410bp), nominal 1 g/kg = gold (BF410cp), nominal 10 g/kg = red (BF410dp), nominal 100 g/kg = brown (BF410ep), consistent with the cap colours of previous JRC-Geel CRMs for GMOs. Each of the bottles was identified by a numbered label indicating the ERM code and the unit number according to filling order. After the inventory and the selection of bottles for future analysis according to a random stratified sampling scheme, the remaining bottles were stored in the dark at 4 ± 3 °C.

Ten randomly selected bottles from each of the powder materials were measured by V-KFT to determine the residual mass fraction of water in the candidate CRMs. The results are summarised in Table 2.

Table 2: Water mass fractions of candidate ERM-BF410p CRMs determined by V-KFT ($N = 10$, $n = 1$). The associated expanded uncertainty (U) has been estimated during validation of the V-KFT method on soya bean powder

Candidate CRM	Water mass fraction [g/kg]	
	\bar{x}	$U (k = 2)$
ERM-BF410ap	18.1	1.1
ERM-BF410bp	11.6	0.7
ERM-BF410cp	24.0	1.4
ERM-BF410dp	19.6	1.2
ERM-BF410ep	20.6	1.2

The particle size distribution in the candidate CRMs was determined based on the deconvoluted laser diffraction pattern of the constituent powders. Five randomly selected bottles from each of the candidate CRMs were analysed twice ($N = 5$, $n = 2$) and 99.93 % of the particles had a size below 1020 μm (Figure 2). The mean particle diameters and standard deviations of the mean, measured by laser diffraction, were 120 μm ($s_x = 4$ μm),

131 μm ($s_x = 10 \mu\text{m}$), 129 μm ($s_x = 12 \mu\text{m}$), 125 μm ($s_x = 25 \mu\text{m}$) and 128 μm ($s_x = 7 \mu\text{m}$) for ERM-BF410ap, bp, cp, dp and ep, respectively.

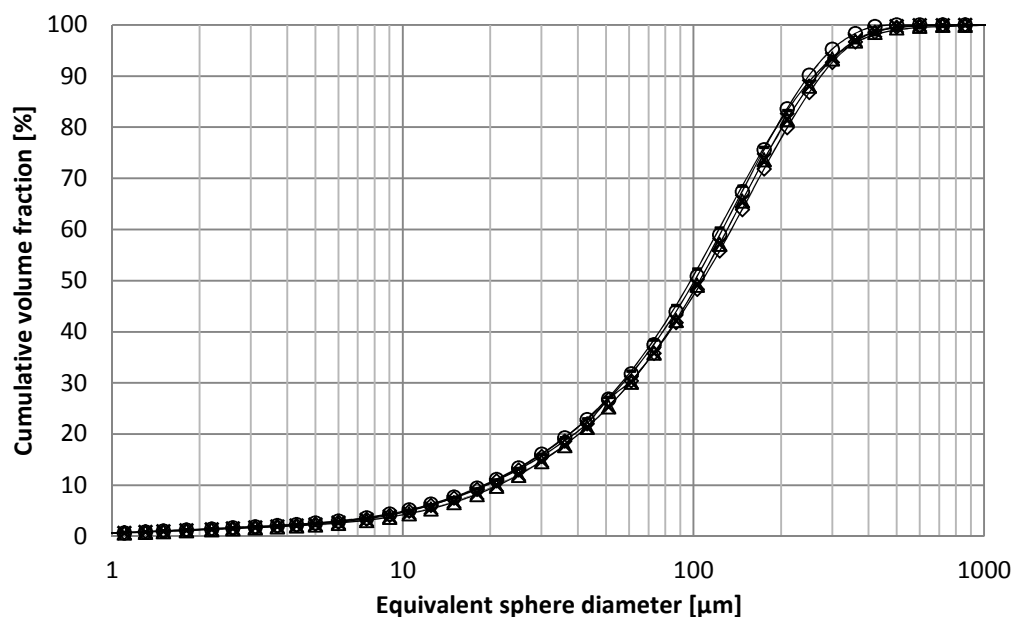


Figure 2: Volume based cumulative distribution of particle size in ERM-BF410ap (\circ), ERM-BF410bp (\diamond), ERM-BF410cp (\triangle), ERM-BF410dp (—) and ERM-BF410ep (\times) analysed by laser diffraction ($N = 5$, $n = 2$). The total particle volume for each preparation is set as 100 %.

3.3 Total DNA content of the powder materials

To investigate if both materials used for the production of ERM-BF410p contain the same mass of DNA, a slight modification of the classical fractionation method developed initially by Ogur and Rosen [11] was employed.

A sequential removal of alcohol-, alcohol-ether- and acid-soluble compounds, followed by acidic extraction with 0.84 mol/L perchloric acid (pH 0.3) at 70 °C was performed. The mass of DNA was determined after derivatisation with diphenylamine using a spectrophotometer. Diphenylamine reacts specifically with 2-deoxyribose linked to purine nucleobases to produce a blue-coloured compound that absorbs at 600 nm [11, 12]. The extractable DNA mass fraction of the two materials was calculated as:

$$\frac{\text{DNA mass extracted from 100 mg GM soya bean powder}}{\text{DNA mass extracted from 100 mg non - GM soya bean powder}}$$

The ratio of the DNA mass extractable from 100 mg of GM and non-GM soya bean powder was found to be (0.761 ± 0.011) ($N = 9$ with an expanded uncertainty, $k = 2$). A t -test has shown a significant difference between the DNA mass extracted from the GM and non-GM powder by the modified Ogur and Rosen [11] method (95 % confidence level).

The assigned certified GM mass values of the prepared mixtures can only be reproduced by real-time PCR, if the different DNA extractability of GM and non-GM soya bean powders is taken into account. The difference in the extractability can be attributed to the difference in the size of the non-GM and GM seeds as observed during their visual inspection. In this context it has to be understood that the ERM-BF410p has been developed to set a common reference point for the implementation of EU legislation on GMO thresholds and labelling.

Gel electrophoresis was used to check the integrity of the DNA. DNA was extracted from 200 mg samples taken from each of the candidate CRM, ERM-BF410ap, ERM-BF410bp,

ERM-BF410cp, ERM-BF410dp and ERM-BF410ep, using a CTAB tip-20 DNA extraction method (Annex A). None of the samples showed DNA degradation (data not shown).

3.4 Consistency measurements

As a control for the gravimetric preparations, the mass fraction of GTS 40-3-2 soya bean event in the mixed materials ERM-BF410cp, ERM-BF410dp and ERM-BF410ep was measured using the in-house validated real-time PCR method provided by Monsanto and published by EURL-GMFF [9].

At the JRC-Geel, genomic DNA was extracted by a validated CTAB extraction method (Annex A) using 200 mg powder samples. After the extraction, the DNA was diluted in a TE-low buffer solution (pH 8.0, 1 mmol/L Tris and 0.01 mmol/L EDTA) and used to produce calibration curves for the soya bean-specific gene and the transgene. The real-time PCR test was calibrated with genomic DNA extracted from pure GTS 40-3-2 soya bean powder. For the calibration curve of the soya bean-specific gene, the DNA was used undiluted (approximately 200 ng DNA per 25 μ L reaction) and diluted up to 200-fold. For the calibration curve of the transgene, the DNA was used in concentration of approximately 50 ng DNA per 25 μ L reaction and was then subsequently diluted up to 1250-fold. The efficiency of the amplification was assessed from the slope of the regression line between the calibrants' mass fractions of GTS 40-3-2 soya bean event and from the C_q-values. The LOD of the PCR method was calculated as 3.3-fold *s* of the lowest calibration point at which *s_{rel}* was below 25 %. The results of the quantification of GTS 40-3-2 soya bean event are shown in Table 3.

The difference in the assigned certified values of the prepared mixtures (Table 8) and GM mass fraction measured by real-time PCR is a consequence of the DNA mass difference of the two base powders (Section 3.3). Although no independent calibration was carried out, the data in Table 3 can be used for confirmation of the processing, but do not necessarily represent the true value of the mass fractions.

Table 3: Quantification of the GTS 40-3-2 soya bean mass fraction in the candidate CRMs by event-specific real-time PCR using genomic DNA from pure GTS 40-3-2 soya bean seed powder for calibration

Candidate CRM	GTS 40-3-2 soya bean mass fraction [g/kg]	<i>U</i> (<i>k</i> = 2) ⁵⁾ [g/kg]
ERM-BF410ap	< 0.09 ^{1) 2)}	-
ERM-BF410bp	994 ¹⁾	16
ERM-BF410cp	0.89 ³⁾	0.03
ERM-BF410dp	8.2 ⁴⁾	0.2
ERM-BF410ep	85.2 ¹⁾	2.3

¹⁾ Mean of 2 samples (extraction replicates) from each of 5 randomly selected bottles (*N* = 5, *n* = 2), with each sample measured in 3 real-time PCR replicates.

²⁾ The value was below the LOD determined during method validation (0.09 g/kg).

³⁾ Mean of 3 samples (extraction replicates) from each of 15 randomly selected bottles (*N* = 15, *n* = 3), with each sample measured in 3 real-time PCR replicates.

⁴⁾ Mean of 2 samples (extraction replicates) from each of 17 randomly selected bottles (*N* = 17, *n* = 2), with each sample measured in 3 real-time PCR replicates.

⁵⁾ Uncertainty of the measurement includes the reproducibility but not the trueness.

4 Homogeneity

A key requirement for any CRM aliquotted into units is the equivalence between those units. In this respect, it is relevant whether the variation between units is significant compared to the uncertainty associated with the certified value, although it is not necessarily relevant whether the variation between units is significant compared to the analytical variation. Consequently, ISO Guide 34:2009 [1] requires RM producers to quantify the between-unit variation. This aspect is covered in between-unit homogeneity studies.

This homogeneity study was planned together with the measurements to control the gravimetric preparations and the short-term stability of the CRMs (Sections 3.4 and 5.1). These data were appropriate for investigating homogeneity since they had been obtained under intermediate precision conditions on bottles taken randomly from the entire batch and analysed in a randomised order. Two extraction replicates per bottle were analysed for ERM-BF410dp and ERM-BF410ep, compared to three for ERM-BF410cp. The number of extraction replicates was chosen based on the intermediate precision of the in-house validated method, such that the standard uncertainty for the within-unit variation would be less than 25 %. Homogeneity of the blank powder was demonstrated in the course of the test for the genetic purity of the raw materials by taking two extraction replicates from 5 randomly selected bottles of ERM-BF410ap. The homogeneity of ERM-BF410bp is related to the purity study of the seeds. As all 201 tested seeds gave a signal for the GTS 40-3-2 soya bean event, using the statistical analysis (Poisson distribution for rare events) with 95 % level of confidence the batch was considered to be homogeneous (Section 3.1).

4.1 Between-unit homogeneity

The between-unit homogeneity was evaluated to ensure that the certified values of the CRMs are valid for all bottles containing the material, within the stated uncertainties.

For the between-unit homogeneity test, the number of bottles selected corresponds to approximately the cube root of the total number of bottles produced. Therefore, 15 bottles were selected for ERM-BF410cp and 17 for ERM-BF410dp. To facilitate both the homogeneity studies and the short-term stability study, 15 bottles were selected for ERM-BF410ep. For each candidate CRM, a random stratified sampling scheme covering the whole batch was used to select the samples. For this, the batch was divided into 15 and 17 groups respectively (with a similar number of bottles) and one bottle was randomly selected from each group. For ERM-BF410cp, three independent samples (extraction replicates) were taken from each bottle whilst for the candidate CRMs with higher mass fractions, ERM-BF410dp and ERM-BF410ep, two independent samples (extraction replicates) were taken from each bottle. All samples were analysed by real-time PCR. Due to the number of PCR plates required, the measurements were performed under intermediate precision conditions. Samples were analysed in a randomised manner to be able to separate a potential analytical trend from a trend in the filling sequence. The results are shown in the figures in Annex B.

Regression analyses were performed to evaluate potential trends in the filling sequence. No trends were observed at a 95 % confidence level.

In addition, regression analyses were performed to evaluate potential trends in the analytical sequence. Some significant trends (95 % confidence level) in the analytical sequence were detected for ERM-BF410cp, indicating a trend in the analytical system. The correction for this trend, even if it is statistically not significant, was found to combine the smallest uncertainty with the highest probability to cover the true value [13]. Correction of trends is therefore expected to improve the sensitivity of the subsequent statistical analysis through a reduction in analytical variation without masking potential between-unit heterogeneities. As the analytical sequence and the unit numbers were not correlated, trends significant on at least a 95 % confidence level were corrected as shown here:

$$x_{i_corr} = x_i - b \cdot i$$

Equation 1

b = slope of the linear regression

i = position of the result in the analytical sequence

The trend-corrected data set for ERM-BF410cp and datasets for ERM-BF410dp and ERM-BF410ep were assessed for consistency using Grubbs outlier tests at a 99 % confidence level on the individual results and on the unit means. No outlying individual results neither unit means were detected using the double Grubbs outlier test.

The trend-corrected data set for ERM-BF410cp and the datasets for ERM-BF410dp and ERM-BF410ep were assessed for consistency using Grubbs outlier tests at a 99 % confidence level on the individual results and on the unit means. No outlying individual results, nor unit means, were detected using the double Grubbs outlier test.

Quantification of between-unit inhomogeneity was undertaken by analysis of variance (ANOVA), which separates the between-unit variation (s_{bb}) from the within-unit variation (s_{wb}). The latter is equivalent to the method intermediate precision if the individual samples were representative of the whole unit.

Evaluation by ANOVA requires mean values per unit which follow at least a unimodal distribution and results for each unit that have approximately the same standard deviation. Too few data are available for the unit means to make a clear statement about the distribution. Therefore, it was visually checked whether all individual data followed a unimodal distribution using histograms and normal probability plots.

It should be noted that $s_{bb,rel}$ and $s_{wb,rel}$ are estimates of the true standard deviations and are therefore subject to random fluctuations. Therefore, the mean squares between groups ($MS_{between}$) can be smaller than the mean squares within groups (MS_{within}), resulting in negative arguments under the square root used for the estimation of the between-unit variation, whereas the true variation cannot be lower than zero. In this case, u_{bb}^* , the maximum inhomogeneity that could be hidden by method intermediate precision, was calculated as described by Linsinger *et al.* [13]. u_{bb}^* is comparable to the LOD of an analytical method, yielding the maximum inhomogeneity that might be undetected by the given study setup.

Method intermediate precision ($s_{wb,rel}$), between-unit standard deviation ($s_{bb,rel}$) and maximum hidden inhomogeneity ($u_{bb,rel}^*$) were calculated as:

$$s_{wb,rel} = \frac{\sqrt{MS_{within}}}{\bar{y}} \quad \text{Equation 2}$$

$$s_{bb,rel} = \frac{\sqrt{\frac{MS_{between} - MS_{within}}{n}}}{\bar{y}} \quad \text{Equation 3}$$

$$u_{bb,rel}^* = \frac{\sqrt{\frac{MS_{within}}{n}} \sqrt[4]{\frac{2}{v_{MS_{within}}}}}{\bar{y}} \quad \text{Equation 4}$$

MS_{within}	within-unit mean square from an ANOVA
$MS_{between}$	between-unit mean square from an ANOVA
\bar{y}	mean of all results of the homogeneity study
n	mean number of replicates per unit
$v_{MS_{within}}$	degrees of freedom of MS_{within}

The results of the evaluation of the between-unit variation are summarised in Table 4.

Table 4: Results of the homogeneity study

Candidate CRM	$s_{wb,rel}$ [%]	$s_{bb,rel}$ [%]	$u_{bb,rel}^*$ [%]	$u_{bb,rel}$ [%]
ERM-BF410cp	14.1	n.c. ¹⁾	4.1	n.c. ¹⁾
ERM-BF410dp	5.7	2.8	2.3	2.8
ERM-BF410ep	4.7	1.8	2.0	2.0

¹⁾ n.c: cannot be calculated as $MS_{between} < MS_{within}$

The homogeneity study showed no outlying unit means or trends in the filling sequence. Therefore, the between-unit standard deviation can be used as an estimate of u_{bb} . As u_{bb}^* sets the limits of the study to detect inhomogeneity, the larger value of s_{bb} and u_{bb}^* is adopted as uncertainty contribution to account for potential inhomogeneity.

4.2 Within-unit homogeneity and minimum sample intake

The within-unit homogeneity is closely correlated to the minimum sample intake. The minimum sample intake is the minimum amount of sample that is representative for the whole unit and thus should be used for analysis. Using sample sizes equal or above the minimum sample intake guarantees the certified value within its stated uncertainty.

Homogeneity and stability experiments were performed using a 200 mg sample intake. This sample intake gives acceptable intermediate precision, demonstrating that the within-unit inhomogeneity no longer contributes to the analytical variation at this sample intake.

ERM-BF410ap and ERM-BF410bp are pure non-GM and GM materials, respectively. Therefore, the minimum sample intake for these materials is not linked to the within-unit homogeneity. However, based on the real-time PCR measurements carried out on these two powders it was concluded that also for these two pure materials the suitable minimum sample intake for real-time PCR is 200 mg.

5 Stability

Time, temperature and light were regarded as the most relevant influences on the stability of the materials. The influence of light was minimised by storing the materials in amber glass bottles which reduce light exposure. In addition, materials were stored in the dark and dispatched in boxes, thus removing any possibility of degradation due to light. Therefore, only the influences of time and temperature needed to be investigated.

Stability testing is necessary to establish the conditions for storage (long-term stability) as well as the conditions for the dispatch of the materials to the customers (short-term stability). During transport, especially in the summer, temperatures of up to 60 °C can be reached and stability under these conditions must be demonstrated, if the samples are to be transported without any additional cooling.

The ERM-BF410ep material was selected for the short-term stability study because it has the highest GM mass fraction, which makes it easier to assess the stability of both powders. The short-term stability study was carried out using an isochronous design [14]. In this approach, samples of ERM-BF410ep were stored for a defined length of time at different temperature conditions. Afterwards, the samples were moved to conditions where further degradation can be assumed to be negligible (reference conditions). At the end of the isochronous storage, the samples were analysed simultaneously under intermediate precision conditions.

ERM-BF410p is a dried soya bean seed powder, which has been prepared in a similar manner to previous GMO CRM soya powders produced by JRC-Geel and which have similar water content and particle size distribution. Therefore, the data obtained from the stability

monitoring of previous soya bean GMO CRMs were used to assess the long-term stability of ERM-BF410p, and to estimate the uncertainty associated with storage of this CRM.

5.1 Short-term stability study

For the short-term stability study, units of ERM-BF410ep were stored at 4 °C, 18 °C and 60 °C for each of 1, 2 and 4 weeks, whereupon they were moved to the reference temperature (-70 °C). Units representing the time point of 0 weeks were kept at a reference temperature (-70 °C). Five units per storage time and temperature were selected using a random stratified sampling scheme. From each unit, two extraction replicates were measured by real-time PCR. The measurements were performed under intermediate precision conditions with respect to the PCR plates, and a randomised sequence was used to differentiate any potential analytical trend from a trend over storage time.

The data were evaluated individually for each of the three temperatures tested. The results were screened for outliers using the single and double Grubbs test at a 99 % confidence level. No statistical outliers were detected in any of the studies for any of the temperatures.

Also, the data were evaluated against storage time, and regression lines of mass fraction versus time were calculated to test for potential increases/decreases of the GTS 40-3-2 soya bean mass fraction due to the simulated shipping conditions. The slopes of the regression lines were tested for statistical significance. There were no trends that were statistically significant on a 95 % confidence level for any of the temperatures.

The material can thus be dispatched without further precautions under ambient conditions.

The results of the measurements are shown in Annex C.

5.2 Long-term stability study

Data from the stability monitoring program for GMO CRMs were available. Previously released soya bean powder CRMs were analysed for their GM mass fraction with 29 data points over a period of 8 years. On each occasion, measurements were performed simultaneously on one PCR plate, using DNA extracted from units stored at the normal storage temperature (4 °C) and at a reference temperature (-70 °C). Each of these studies can be viewed as a two-point isochronous study. The evaluation was based on the GM mass fraction ratio of results of the samples stored at 4 °C and -70 °C.

To verify that the data obtained from stability monitoring of other soya bean GMO CRMs produced and stored in the same way as ERM-BF410p, can be used to estimate the stability uncertainty contribution for ERM-BF410p, the data of the 4 °C short-term stability study (Section 5.1) were compared to the stability monitoring data. The outcome did not contradict the conclusions drawn from the long-term stability study on the uncertainty contribution relating to the storage of the CRM.

The long-term stability data were screened for outliers using the single and double Grubbs test at a confidence level of 99 %. No statistical outliers were detected, and the results were retained for the estimation of u_{ITS} .

The data were also evaluated against storage time and regression lines were calculated. The slopes of the regression lines were tested for statistical significance (loss/increase due to storage). No significant trend was detected at a 95 % confidence level.

The material can, therefore, be stored at 4 °C.

The results of the measurements are shown in Annex D.

5.3 Estimation of uncertainties

Due to the intrinsic variation of measurement results, no study can entirely rule out the degradation of materials, even in the absence of statistically significant trends. It is, therefore,

necessary to quantify the potential degradation that could be hidden by the method intermediate precision, i.e. to estimate the uncertainty of stability. This means that, even under ideal conditions, the outcome of a stability study can only be that there is no detectable degradation.

The uncertainties of stability during dispatch and storage were estimated, as described in [15]. In this approach, the uncertainty of the linear regression line with a slope of zero was calculated. The uncertainty contributions u_{sts} and u_{lts} were calculated as the product of the chosen transport time/shelf life and the uncertainty of the regression lines as:

$$u_{sts,rel} = \frac{s_{rel}}{\sqrt{\sum (t_i - \bar{t})^2}} \cdot t_{tt} \quad \text{Equation 5}$$

$$u_{lts,rel} = \frac{s_{rel}}{\sqrt{\sum (t_i - \bar{t})^2}} \cdot t_{sl} \quad \text{Equation 6}$$

s_{rel}	relative standard deviation of all results of the stability study
t_i	time elapsed at time point i
\bar{t}	mean of all t_i
t_{tt}	chosen transport time (1 week at 60 °C)
t_{sl}	chosen shelf life (24 months at 4 °C)

The following uncertainties were estimated:

- $u_{sts,rel}$, the uncertainty of degradation during dispatch. This was estimated from the 60 °C studies. The uncertainty describes the possible change during a dispatch at 60 °C lasting for 1 week.
- $u_{lts,rel}$, the stability during storage. This uncertainty contribution was estimated from the stability monitoring program for soya bean GMO CRMs. The uncertainty contribution describes the possible degradation during 24 months storage at 4 °C.

The results of these evaluations are summarised in Table 5.

Table 5: Uncertainties of stability during dispatch and storage. $u_{sts,rel}$ was calculated for a temperature of 60 °C and 1 week; $u_{lts,rel}$ was calculated for a storage temperature of 4 °C and 24 months

Candidate CRM	$u_{sts,rel}$ [%]	$u_{lts,rel}$ [%]
ERM-BF410p	0.5	1.1

After the certification study, the materials will be included in the JRC-Geel's regular stability monitoring programme to assess their further stability.

6 Characterisation

For the purpose of RM certification, material characterisation is the term used to describe the process of determining the certified value of a reference material.

The five candidate CRMs, under the label ERM-BF410p, are soya bean powder materials processed from non-GM and GM seeds. While ERM-BF410ap was prepared from the pure non-GM material and ERM-BF410bp from the pure GM material, the other candidate CRMs of the ERM-BF410p series are gravimetrically diluted mixtures of the pure non-GM and GM soya bean seed powders. ERM-BF410p is certified for the mass fraction of GTS 40-3-2 soya bean event. Gravimetric mixing was the method of choice based on a primary method of measurement confirmed by PCR analysis.

6.1 Genetic purity of the materials

The genetic purity with respect to the GTS 40-3-2 soya bean event of the GM and non-GM batches used for the processing of the candidate CRMs was investigated to calculate the certified value.

No indication was found that the GM soya bean material contained seeds that were negative for the event GTS 40-3-2 soya bean (Section 3.1).

The powder used for the production of ERM-BF410ap did not contain traces of GTS 40-3-2 soya bean above the LOD of the real-time PCR method used (Sections 3.1 and 3.4). The certified value for ERM-BF410ap is therefore based on the LOD of the real-time PCR method, as determined during in-house method validation.

The eventual adventitious presence of other GM events in both the GM and non-GM soya bean powders was verified by using a real-time PCR-based ready-to-use multi-target analytical system for GM detection developed by JRC-Ispra [16]. This test was performed at the JRC-Geel by using a pre-spotted 96-well plate containing primers and probes for simultaneous detection of 13 GM specific events (A2704, A5547, CV127, DAS68416, DP305423, DP356043, FG72, GTS 40-3-2, MON87701, MON87705, MON87708, MON87769 and MON89788) and the primers and probes for the specific detection of the lectine (Lec), the soya bean reference gene. Any stacked events derived from the single-insert GMOs included in the system would also be detected.

The results indicated that both soya bean powders used for the production of ERM-BF410p did not contain any of the above tested GM events and were only positive for the taxon-specific detection for soya bean (Lec).

Since no evidence of contamination was found in the non-GM and the GM materials, 100 % genetic purity was used for the calculation of the certified mass fraction of GTS 40-3-2 soya bean in the powder mixtures. The difference between the statistically established genetic purity of at least 98.5 % (Section 3.1) and the 100 % genetic purity was taken into account in the uncertainty calculation.

6.2 Mass fractions and their uncertainties

The certified mass fraction values are based on the mass fractions of mixed GM and non-GM powder, corrected for their water mass fractions and taking into account the powder's genetic purity with regards to the GTS 40-3-2 soya bean event. The values were calculated according to the following equations:

$$\text{GM mass fraction [g/kg]} = \frac{m_{\text{GM,dry}}}{m_{\text{GM,dry}} + m_{\text{nonGM,dry}}} \times 1000 \quad \text{Equation 7}$$

$$m_{\text{GM,dry}} = m_{\text{GM}} \times (1 - \text{WMF}_{\text{GM}}) \quad \text{Equation 8}$$

$$m_{\text{nonGMdry}} = m_{\text{nonGM}} \times (1 - \text{WMF}_{\text{nonGM}}) \quad \text{Equation 9}$$

$m_{\text{GM,dry}}$	mass [g] of the GM powder corrected for its water mass fraction
$m_{\text{nonGM,dry}}$	mass [g] of the non-GM powder corrected for its water mass fraction
m_{GM}	mass [g] of the GM powder used for the dilution
m_{nonGM}	mass [g] of the non-GM powder used for the dilution
WMF_{GM}	water mass fraction of the GM powder [g/g]
$\text{WMF}_{\text{nonGM}}$	water mass fraction of the non-GM powder [g/g]

The data supporting the calculation of the mass fractions of GTS 40-3-2 soya bean are summarised in Table 6.

Table 6: Subsequent mixing of pure GTS 40-3-2 GM soya bean seed powder with pure non-GM powder to prepare the ERM-BF410cp, dp and ep materials

Candidate CRM	GM powder ¹⁾			Non-GM powder ¹⁾		Mixtures
	GM Mass fraction [g/kg]	Water mass fraction $\pm U(k=2)$ [g/kg]	Mass [g]	Water mass fraction $\pm U(k=2)$ [g/kg]	Mass [g]	Calculated GM mass fraction [g/kg]
ERM-BF410ep	1000.0 ²⁾	15.5 ± 1.0	495.32	25.6 ± 1.7	4504.62	100
ERM-BF410dp	100.0 ³⁾	24.9 ± 1.6	499.64	25.6 ± 1.7	4500.31	10.0
ERM-BF410cp	10.0 ⁴⁾	25.6 ± 1.7	499.86	25.6 ± 1.7	4500.03	1.00

¹⁾ Calculations of the mass fraction of GTS 40-3-2 soya bean in the powder mixtures are based on a 100 % genetic purity with regard to GTS 40-3-2 soya bean of the non-GM and GM powder materials.

²⁾ Pure GTS 40-3-2 GM soya bean seed powder was used for the preparation of ERM-BF410ep.

³⁾ GM powder mixture ERM-BF410ep was used for the preparation of ERM-BF410dp.

⁴⁾ GM powder mixture ERM-BF410dp was used for the preparation of ERM-BF410cp.

The uncertainties of the certified GTS 40-3-2 soya bean mass fractions (u_{char}) have several components, i.e. the uncertainty arising from weighing ($u_{\text{char},1}$), the uncertainty of the determination of the water mass fraction ($u_{\text{char},2}$), and the uncertainties associated with the determination of the genetic purity concerning the GTS 40-3-2 soya bean event of the non-GM and GM powder materials ($u_{\text{char},3}$ and $u_{\text{char},4}$, respectively). Based on a statistical analysis of the probability distribution of finding a negative seed in the GM raw material, it was concluded that the genetic purity of the event GTS 40-3-2 soya bean event in this CRM, was higher than 98.5 % (95 % confidence level, Section 3.1). This value was taken into account when estimating the uncertainty of the certified value (Table 7).

Table 7: Uncertainty budgets for the mass fractions of GTS 40-3-2 soya bean in ERM-BF410p

Candidate CRM	Nominal mass fraction [g/kg]	Standard uncertainty contribution [g/kg]				Combined standard uncertainty u_{char} [g/kg]
		$u_{\text{char},1}$ ¹⁾	$u_{\text{char},2}$ ²⁾	$u_{\text{char},3}$ ³⁾	$u_{\text{char},4}$ ⁴⁾	
ERM-BF410ap	0	n.a. ⁵⁾	n.a. ⁵⁾	0.0260	n.a. ⁵⁾	0.0260
ERM-BF410bp	1000	n.a. ⁵⁾	n.a. ⁵⁾	n.a. ⁵⁾	4.3014	4.3014
ERM-BF410cp	1	0.0006	0.0017	0.0260	0.0043	0.0264
ERM-BF410dp	10	0.0046	0.0144	0.0260	0.0430	0.0525
ERM-BF410ep	100	0.0328	0.1177	0.0260	0.4301	0.4479

¹⁾ Standard uncertainty of the mass determination, based primarily on the uncertainty of the balance and the number of weighing steps required.

²⁾ Standard uncertainty of the water mass fraction determination by V-KFT.

³⁾ Standard uncertainty of the genetic purity estimation of the non-GM powder material (LOD = 0.09 g/kg), based on the half-width of the interval between 0 and 0.09 g/kg, divided by the square root of 3 (rectangular distribution).

⁴⁾ Standard uncertainty of the genetic purity estimation of the GM raw material (> 98.5 %), based on the interval between 98.5 % and 100 % divided by the square root of 3 (rectangular distribution).

⁵⁾ not applicable

6.3 Consistency measurements

Real-time PCR measurements confirmed that no mixing errors were made during the preparation of the candidate CRMs (Section 3.4). Additionally, gel electrophoresis proved that the DNA was not degraded during the processing of the candidate CRMs (Section 3.3).

7 Value Assignment

Certified values are values that fulfil the highest standards of accuracy assessment. Therefore full uncertainty budgets in accordance with the 'Guide to the Expression of Uncertainty in Measurement' [4] were established.

The assigned certified values are based on the masses of dried powder of GM seeds and non-GM seeds used in the gravimetrical preparation. The masses of the powders were corrected for their respective water mass fractions during the preparation of the materials (Table 6).

The assigned uncertainty consists of uncertainties relating to characterisation, u_{char} (Section 6.2), potential between-unit inhomogeneity, u_{bb} (Section 4.1), and potential degradation during transport, u_{sts} , and long-term storage, u_{its} (Section 5.3). These different contributions were combined to estimate the relative expanded uncertainty of the certified value ($U_{\text{CRM,rel}}$) with a coverage factor k given as:

$$U_{\text{CRM,rel}} = k \cdot \sqrt{u_{\text{char,rel}}^2 + u_{\text{bb,rel}}^2 + u_{\text{sts,rel}}^2 + u_{\text{its,rel}}^2} \quad \text{Equation 10}$$

- u_{char} was estimated as described in Section 6.2.
- u_{bb} was estimated as described in Section 4.1.
- u_{sts} and u_{its} were estimated as described in Section 5.3.

For the blank material, the LOD of the method was used to describe the 95 % confidence interval of the certified mass fraction of the event (< 0.09 g/kg). This was supported by the high genetic purity with regards to the GTS 40-3-2 soya bean event of the non-GM material and the absence of a mixing step; calculating the U_{CRM} for the blank material on the basis of the only quantifiable standard uncertainty ($u_{\text{char},3}$) gives a value of $U = 0.06$ g/kg (assuming $k = 2$), which is below the certified < 0.09 g/kg value. The LOD is, therefore, a conservative estimate of the certified value and its uncertainty.

For the pure GM material, the statistically calculated genetic purity of the GM seed batch (Section 3.1) was used to describe the 95 % confidence interval of the certified mass fraction of the event (> 985 g/kg). Calculating U_{CRM} for the pure GM material on the basis of the only quantifiable standard uncertainty ($u_{\text{char},4}$) gives a value of $U = 9$ g/kg (assuming $k = 2$), which is less than the difference between the nominal value (1000 g/kg) and the certified value (> 985 g/kg). The statistically calculated genetic purity is, therefore, a conservative estimate of the certified value and its uncertainty.

For the three mixtures, the certified values were established by gravimetry, and the measured mass fraction values had an expanded uncertainty with a coverage factor of 2, established during calibration of the balance. Therefore, the same coverage factor ($k = 2$) was used to obtain the expanded uncertainties for ERM-BF410cp, dp and ep.

The certified values and their uncertainties are summarised in Table 8.

Table 8: Certified values and their uncertainties for ERM-BF410p

CRM	Certified value [g/kg]	u_{char} [g/kg]	u_{bb} [g/kg]	u_{sts} [g/kg]	u_{its} [g/kg]	$U_{\text{CRM}}^{3)}$ [g/kg]
ERM-BF410ap	< 0.09 ¹⁾	0.0260	n.a. ⁴⁾	n.a. ⁴⁾	n.a. ⁴⁾	-
ERM-BF410bp	> 985 ²⁾	4.3014	n.a. ⁴⁾	n.a. ⁴⁾	n.a. ⁴⁾	-
ERM-BF410cp	1.00	0.0264	0.0410	0.0050	0.0110	0.10
ERM-BF410dp	10.0	0.0525	0.2800	0.0500	0.1100	0.6
ERM-BF410ep	100	0.4479	1.9998	0.4999	1.0999	5

¹⁾ With 95 % confidence, the certified value is below this level.

²⁾ With 95 % confidence, the certified value is above this level.

³⁾ Expanded ($k = 2$) and rounded uncertainty

⁴⁾ not applicable

8 Metrological traceability and commutability

8.1 Metrological traceability

Identity

The certified identity is based on the documentary traceability to the GTS 40-3-2 soya bean event, (Biosafety Clearing House, record ID 14796) [8].

Quantity value

The traceability chain for ERM-BF410cp, dp and ep is based on the use of calibrated balances and a thorough control of the weighing procedure.

The certified values for the pure non-GM and GM CRMs, ERM-BF410ap and ERM-BF410bp, respectively, are based on the genetic purity assessment using event-specific GTS 40-3-2 soya bean real-time PCR.

The certified values are therefore traceable to the International System of Units (SI).

8.2 Commutability

ERM-BF410p was prepared gravimetrically from non-GM and GM seed powders with the aim to resemble as much as possible the kind of thresholds set in the corresponding EU legislation for food and feed, namely mass fractions.

ERM-BF410p is intended to be used as calibrant for real-time PCR measurements of the soya bean GM event GTS 40-3-2 in food and feed. Consequently, this certified reference material is establishing, together with the measurement method validated by the EURL-GMFF [9], the arbitrary reference system required for quantification of GTS 40-3-2 soya bean. Therefore, commutability, which is a crucial characteristic for reference materials in case that different measurement method would be applied, does not have to be considered here.

9 Instructions for use

9.1 Safety and protection of the environment

The usual laboratory safety measures apply. The material is for *in-vitro* use only. As it is a milled material, it does not contain any viable seeds.

9.2 Storage conditions

The materials should be stored at 4 ± 3 °C in the dark. Care should be taken to avoid any change of the moisture content once the units are open, as the material is hygroscopic. The user should close CRM bottles immediately after taking a sample.

Please note that the European Commission cannot be held responsible for changes that happen during storage of the material at the customer's premises, especially for opened bottles.

9.3 Minimum sample intake

The minimum sample intake for a DNA extraction is 200 mg soya bean powder.

ERM-BF410ap and ERM-BF410bp are pure non-GM and GM materials. Therefore, the minimum sample intake for these materials is not linked to the within-unit homogeneity. Nevertheless it is recommended that the same sample intake is used as for the mixed materials to obtain a significant amount of DNA.

9.4 Use of the certified value

The main purpose of these materials is for calibration or quality control of GTS 40-3-2 soya bean event identification and quantification methods. As with any reference material, they can be used for establishing control charts and validation studies.

The user is reminded that this reference material is certified for its GTS 40-3-2 soya bean mass fraction and should be used for measurements expressed in mass fractions. The exact relationship between the certified GM powder mass fractions and the corresponding DNA copy number ratio is not known. Changing the measurement unit from mass fraction to copy number per haploid genome equivalent, for instance, requires the use of a conversion factor

that is only an approximate value, thereby adding additional uncertainty to the measurement result.

Use as a calibrant

If this matrix material is used as calibrant, the uncertainty of the certified value shall be taken into account in the estimation of the measurement uncertainty. Furthermore, it should be noted that using the same material for calibration and quality control limits the effectiveness of the control, as calibrant and quality control material are based on the same raw materials. If this is unavoidable, it is recommended that different mass fraction levels of ERM-BF410p are used for calibration and for quality control.

Comparing an analytical result with the certified value

A result is unbiased if the combined standard uncertainty of measurement and certified value covers the difference between the certified value and the measurement result (see also ERM Application Note 1, www.erm-crm.org [17]).

When assessing the method performance, the measured values of the CRMs are compared to the certified values. The procedure is summarised here:

- Calculate the absolute difference between mean measured value and the certified value (Δ_{meas}).
- Combine the measurement uncertainty (u_{meas}) with the uncertainty of the certified value (u_{CRM}): $u_{\Delta} = \sqrt{u_{\text{meas}}^2 + u_{\text{CRM}}^2}$
- Calculate the expanded uncertainty (U_{Δ}) from the combined uncertainty (u_{Δ}), using an appropriate coverage factor, corresponding to a level of confidence of approximately 95 %.
- If $\Delta_{\text{meas}} \leq U_{\Delta}$ then no significant difference exists between the measurement result and the certified value, at a confidence level of approximately 95 %.

Use in quality control charts

The materials can be used for quality control charts. Using CRMs for quality control charts has the added value that a trueness assessment is built into the chart.

Acknowledgments

The authors would like to acknowledge the support received from Patrick Conneely and Marie France Tumba-Tshilumba from JRC-Geel during the processing of this CRM.

Furthermore, the authors would like to thank Håkan Emteborg (JRC-Geel) and Liesbet Deprez (JRC-Geel) for their constructive comments and reviewing of this certification report.

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Annexes

Annex A: CTAB-tip20 DNA extraction method (as modified in-house)

Solutions and reagents

1. CTAB buffer A
 - 1 % (w/v) CTAB
 - 0.7 M NaCl
 - 0.1 M Tris-HCl, pH 8.0
 - 15 mM Na₂EDTA, pH 8.0
2. CTAB buffer B
 - 1 % (w/v) CTAB
 - 0.1 M Tris-HCl, pH 8.0
 - 15 mM Na₂EDTA, pH 8.0
3. 2-mercaptoethanol
4. Chloroform-Octanol (24:1)
5. 1.2 M NaCl
6. 1 mM Tris, pH 8.0
7. 0.01 mM Na₂EDTA, pH 8.0
8. Proteinase K, 20 mg/mL
9. RNase A, 100 mg/mL
10. Qiagen Genomic-tip20 columns
11. Qiagen QBT equilibration buffer
12. Qiagen QC washing buffer
13. Qiagen QF elution buffer
14. Isopropanol
15. Ethanol
16. 70 % (v/v) Ethanol
17. TE low buffer, 1 mmol/L Tris and 0.01 mmol/L EDTA, pH 8.0

DNA extraction protocol

- a) Weigh 200 mg soya bean powder into a 2 mL microcentrifuge tube
- b) Add 1.3 mL of CTAB Buffer A 1% + 5 μ L RNase A + 6.5 μ L Proteinase K + 26 μ L 2-mercaptoethanol and mix by vortexing
- c) Incubate 1 h at 65 °C, shaking at 1400 rpm
- d) Centrifuge for 10 min at 16000 x *g* at RT
- e) Transfer 750 μ L of supernatant to a 2 mL microcentrifuge tube containing 1 mL of chloroform:octanol (24:1)
- f) Mix thoroughly by inverting, incubate 5 min at room temperature (RT)
- g) Centrifuge for 10 min at 16000 x *g* at RT
- h) Transfer 600 μ L of supernatant to a new 2 mL microcentrifuge tube containing 700 μ L of CTAB Buffer B
- i) Mix thoroughly by inverting, incubate 30 min at RT
- j) Centrifuge for 20 min at 16000 x *g* at RT
- k) Discard the supernatant by pipetting and conserve the pellet
- l) Add 200 μ L of 1.2 M NaCl
- m) Incubate 5 min at 50 °C, shaking at 1400 rpm
- n) Add 1.6 mL of G2 buffer + 2.5 μ L of RNase A + 20 μ L of Proteinase K
- o) Incubate 1 h at 50 °C, shaking at 500 rpm
- p) Centrifuge 5 min at 16000 x *g* at RT
- q) Equilibrate a Qiagen Genomic-tip 20/G column with 1 mL of QBT buffer
- r) Apply the sample to the equilibrated Genomic-tip 20/G column by pipetting
- s) Wash the genomic-tip 20/G column with 3 mL of QC buffer
- t) Elute the genomic DNA with 1 mL of QF buffer (pre-warmed at 50 °C) and collect the DNA in a 2 mL tube
- u) Add 700 μ L of isopropanol to each tube, invert 10 times
- v) Centrifuge for 30 min at 10000 x *g* at 4 °C, discard the supernatant by pipetting
- w) Wash the pellet with 1 mL of 70 % ethanol
- x) Centrifuge 10 min at 13000 x *g* at 4 °C
- y) Discard the supernatant by pipetting and air-dry the pellet for 10 min
- z) Dissolve the DNA pellet in 80 μ L of TE Low buffer preheated at 50 °C, incubate 10 min at 50 °C while shaking at 500 rpm. Let the pellet to dissolve completely overnight at RT and store the samples at + 4 °C (short term) or at -20 °C (long term).

Annex B: Results of the homogeneity measurements

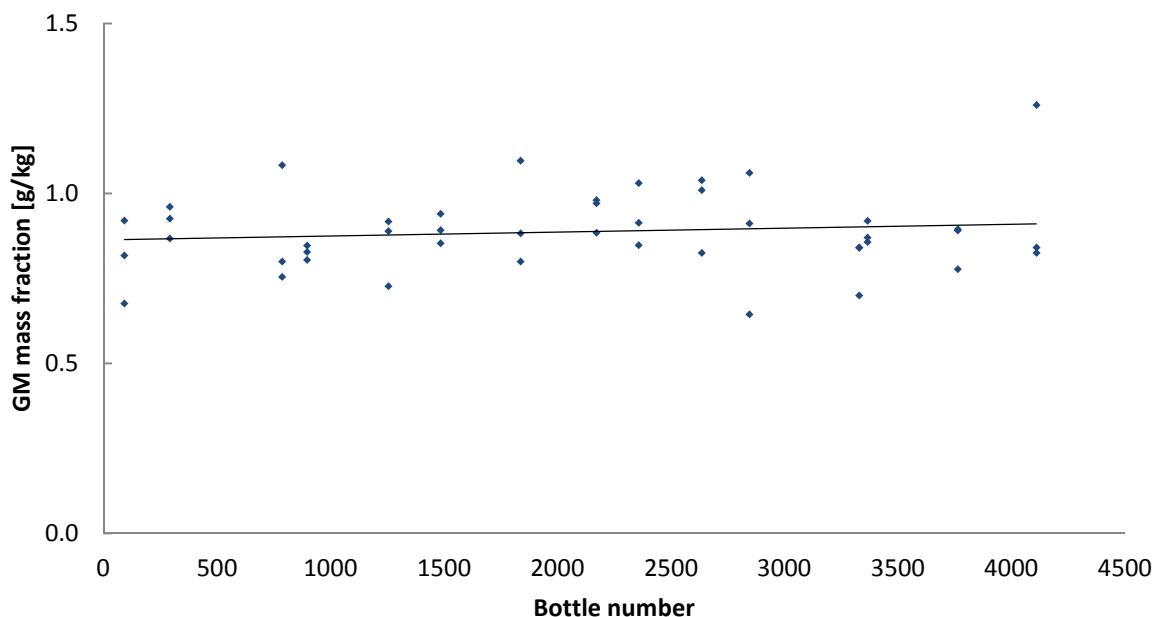


Figure B1: Real-time PCR measurement results for ERM-BF410cp. Three samples (extraction replicates) were measured from each of 15 randomly selected bottles ($N = 15$, $n = 3$), with each sample measured in 3 real-time PCR replicates under intermediate precision conditions. The straight line is a least-squares linear regression for all data points.

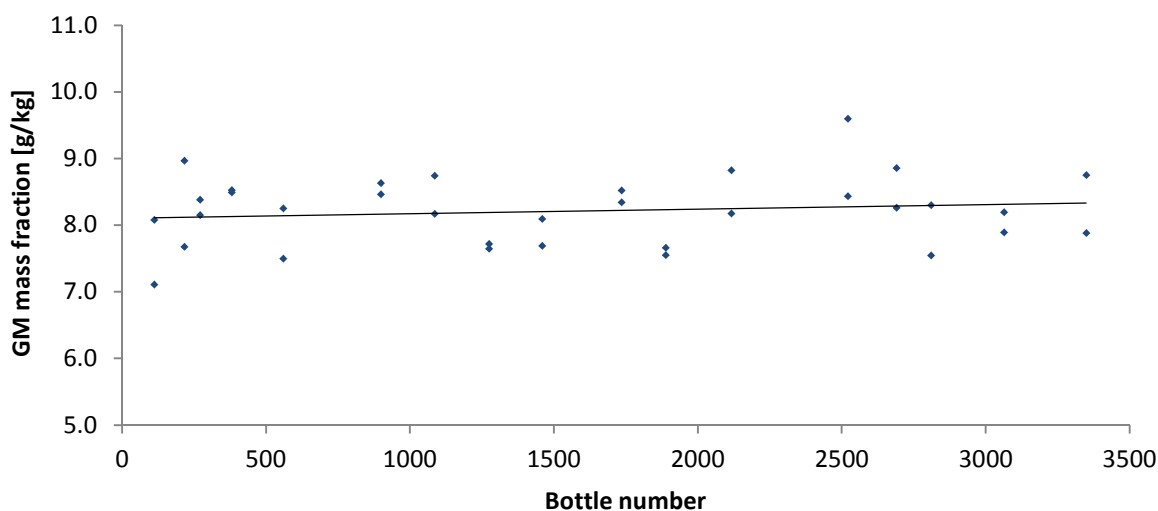


Figure B2: Real-time PCR measurement results for ERM-BF410dp. Two samples (extraction replicates) were measured from each of 17 randomly selected bottles ($N = 17$, $n = 2$), with each sample measured in 3 real-time PCR replicates under intermediate precision conditions. The straight line is a least-squares linear regression for all data points.

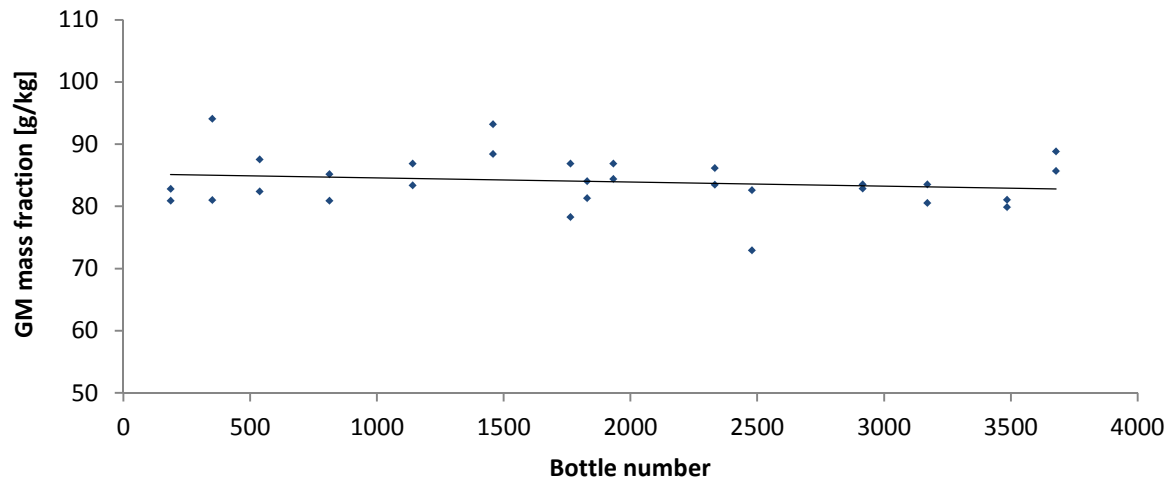


Figure B3: Real-time PCR measurement results for ERM-BF410ep. Two samples (extraction replicates) were measured from each of 15 randomly selected bottles ($N = 15$, $n = 2$), with each sample measured in 3 real-time PCR replicates under intermediate precision conditions. The straight line is a least-squares linear regression for all data points.

Annex C: Results of the short-term stability measurements

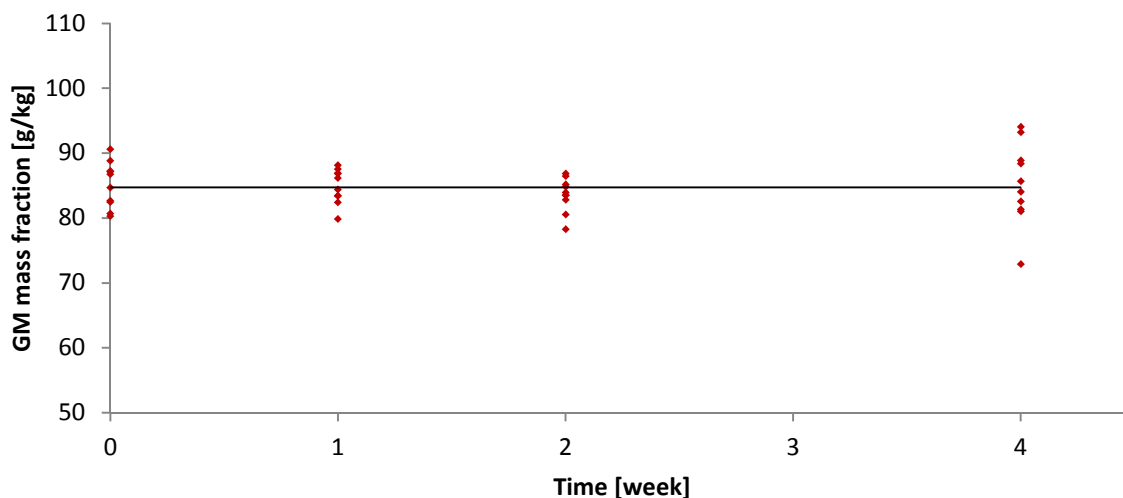


Figure C1: Real-time PCR measurement results for ERM-BF410ep during short-term stability testing at 4 °C. For each storage time, 2 samples (extraction replicates) were measured from each of 5 randomly selected bottles ($N = 5$, $n = 2$), with each sample measured in 3 real-time PCR replicates under intermediate precision conditions. The straight line is a least-squares linear regression for all data points.

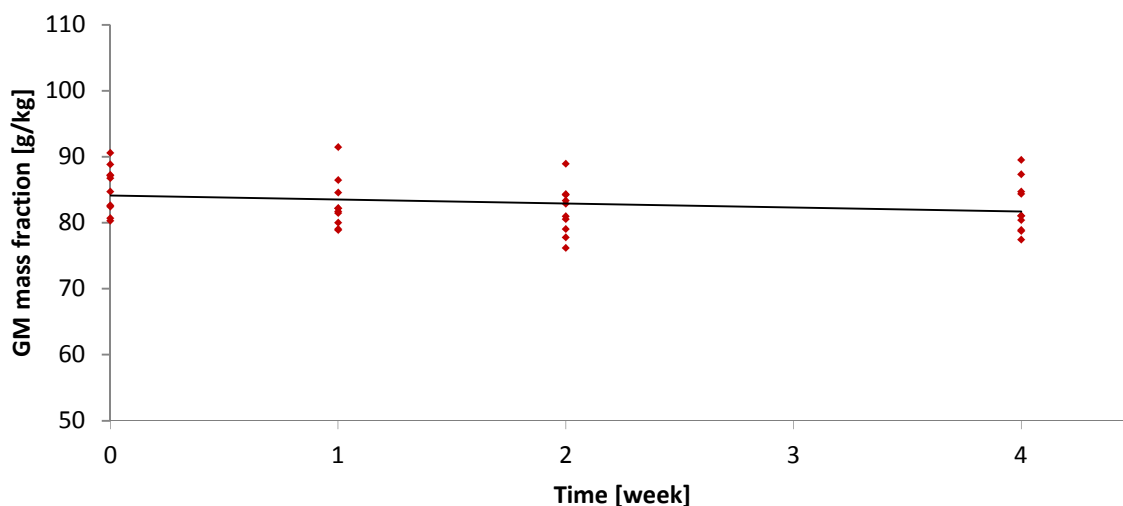


Figure C2: Real-time PCR measurement results for ERM-BF410ep during short-term stability testing at 18 °C. For each storage time, 2 samples (extraction replicates) were measured from each of 5 randomly selected bottles ($N = 5$, $n = 2$), with each sample measured in 3 real-time PCR replicates under intermediate precision conditions. The straight line is a least-squares linear regression for all data points.

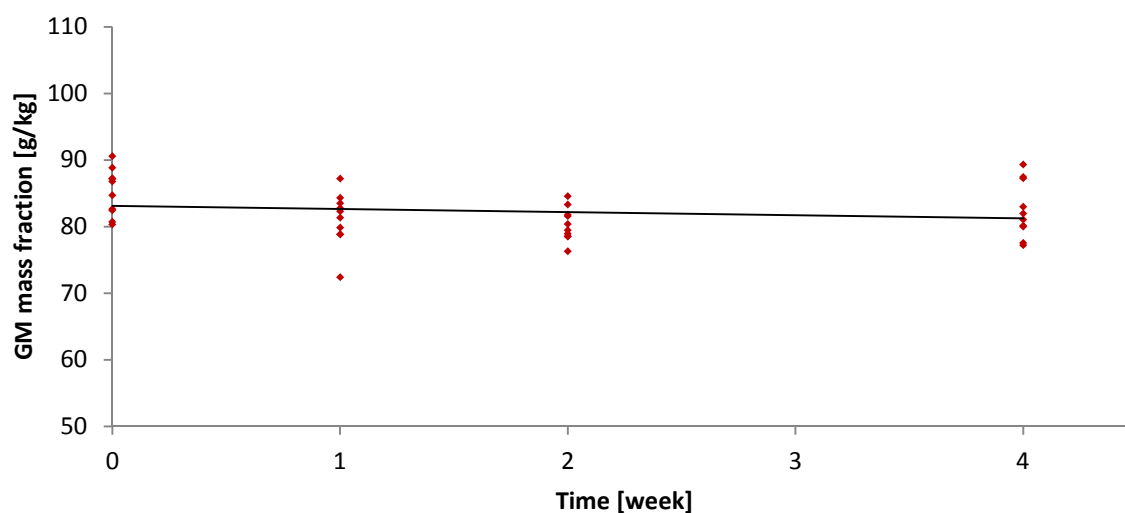


Figure C3: Real-time PCR measurement results for ERM-BF410ep during short-term stability testing at 60 °C. For each storage time, 2 samples (extraction replicates) were measured from each of 5 randomly selected bottles ($N = 5$, $n = 2$), with each sample measured in 3 real-time PCR replicates under intermediate precision conditions. The straight line is a least-squares linear regression for all data points.

Annex D: Results of the long-term stability measurements

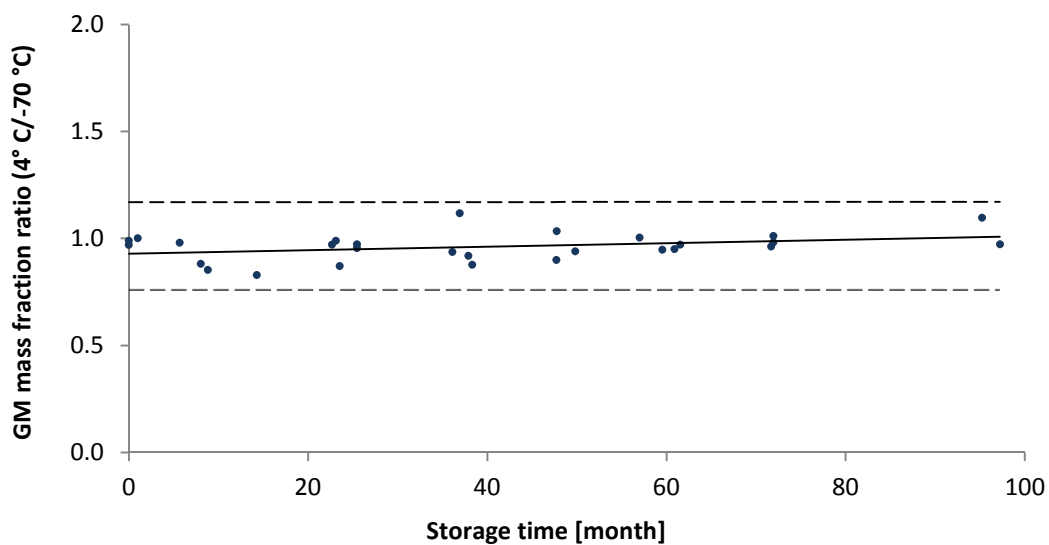


Figure D1: Real-time PCR measurement results of ERM-BF410ep (1, 2 and 4 weeks) compared to ERM-BF410k, ERM-BF410n, ERM-BF425, ERM-BF426, ERM-BF432 and ERM-BF437 (data from the post-certification monitoring). The dashed lines give the limits of 3s obtained for the measurement results. The straight line is a least-squares linear regression for all data points.

European Commission

EUR 28665 EN – Joint Research Centre – Directorate F – Health, Consumers and Reference Materials

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Author(s): B. Dimitrievska, A.M. Kortekaas, J. Seghers, H. Leys, Ph. Corbisier, S. Trapmann

Luxembourg: Publications Office of the European Union

2017 – 34 pp. – 21.0 x 29.7 cm

EUR – Scientific and Technical Research series – ISSN 1831-9424

ISBN 978-92-79-70107-8

doi: 10.2760/341185

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